Supplemental Figure Legends

Figure S1 Topology of A17. Related to Figure 1.
(A) Diagram of A17 depicting the insertion of A17 in the membrane and residues mutated at proposed sites that are exposed on the cytoplasmic side of the membrane. WT A17 has Cys residues at positions 101, 121 and 178 of which only Cys178 was predicted to be exposed. Several mutated A17 proteins were engineered in which either the natural Cys residues were mutated to Ala or the amino acids at positions 38, 107, 108 or 109 were mutated to Cys. (B) Cells were infected with a recombinant VACV (vIndA17) in which the endogenous A17 gene was repressed and transfected with plasmids encoding mutated A17. After 18 h, the cells were treated with digitonin, to permeabilize the plasma membrane while leaving the ER intact, and the membrane insoluble cross-linker maleimide-PEG 2000 (MalPEG). Lysates were solubilized with SDS and analyzed by Western blotting with antibody to A17 to reveal the two bands representing the processed and unprocessed forms of A17 and the slightly slower migrating upper band modified by MalPEG. Only the two bands representing the unmodified forms of A17 were seen when all three Cys of A17 were changed to Ala (lanes 5, 6) or only Cys178 (lane 10) of WT A17 was mutated. In contrast the additional MalPEG-modified band was seen with WT A17 (lane 1); WT A17 with an additional Cys at position 107 (lane 2); A17 with the three natural Cys removed and Cys added at position 38 (lane 3), 108 (lane 4) or 109 (lane 5). The absence of a modified band with Cys107 (lane 8) was possibly due to close proximity to the membrane. The modification of amino acids 38,108, 109 and 178 supported the topology model in panel A.

Figure S2 Codon Optimized A17. Related to Figure 1.
(A) E. coli codon optimized nucleotide sequence of VACV A17. The translation start site is underlined. (B) The A17 protein sequence with original translation start site underlined and preceding 6 histidines in italics and thrombin cleavage site in bold.

Figure S3 Diameters and Lengths of tubules. Related to Figure 2
(A) Density plot of tubule and vesicle diameters. EM micrographs from several experiments were measured using ImageJ and plotted with R using the density plot function in ggplot. 40 to 70 vesicles or tubules were measured for His-A17 lipids and lipid alone. Diameters in nanometers are plotted on the x-axis and gaussian kernel density estimates are plotted on the y-axis as described http://docs.ggplot2.org/0.9.3.1/geom_density.html. (B) Box plots. Lengths of tubules formed with Low (0.25 mg), Medium (0.5 mg) and High (1.0 mg) of His-A17 and 1.0 mg of lipids (w/w ratio).

Movie S1 Tomography. Related Figure 4, Row 1

Movie S2 Tomography. Related to Figure 4, Row 2.

Movie S3 Tomography. Related to Figure 4, Row 3. Arrows point to connections between the nuclear envelope and aggregated ER.

Figure S1

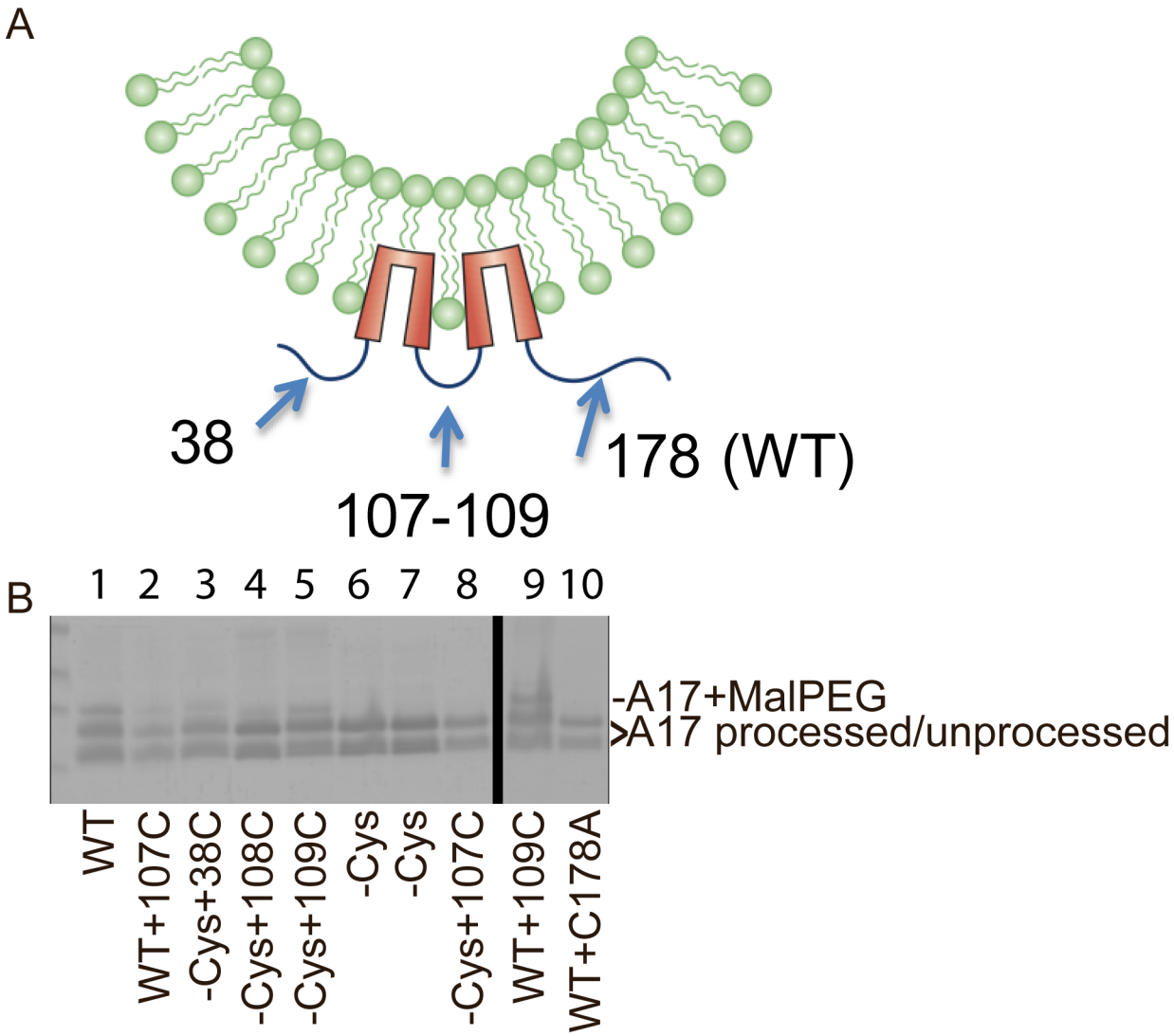

A
38
107-109
178 (WT)
B
1 2 3 4 5 6 7 8 9 10
-A17+MalPEG
>A17 processed/unprocessed
WT
WT+107C
-Cys+38C
-Cys+108C
-Cys+109C
-Cys
-Cys
-Cys+107C
WT+109C
WT+C178A

Figure S2

(A)

TTAATTAAGGATCCGCTGGTTCCGCGTGGTAGCATGAGCTATCTGCGCTATTATAATATG

CTGGATGATTTTAGCGCTGGTGCCGGTGTTCTGGATAAAGACCTGTTTACCGAAGAACAG

CAGCAGAGCTTTATGCCGAAAGATGGTGGTATGATGCAGAATGATTATGGTGGCATGAAT

GATTATCTGGGCATTTTTAAAAATAATGATGTGCGTACCCTGCTGGGTCTGATTCTGTTT

GTTCTGGCCCTGTATTCTCCTCCGCTGATTAGCATTCTGATGATTTTTATTAGCAGCTTT

CTGCTGCCGCTGACCAGCCTGGTTATTACCTATTGTCTGGTGACCCAGATGTATCGTGGT

GGTAATGGTAATACCGTGGGTATGAGCATTGTTTGTATTGTTGCAGCCGTGATTATTATG

GCCATTAATGTGTTTACCAATAGCCAGATTTTTAACATCATTAGCTATATTATCCTGTTT

ATTCTGTTTTTTGCCTATGTGATGAATATTGAACGCCAGGATTATCGTCGTAGCATTAAT

GTTACCATTCCGGAACAGTATACCTGCAATAAACCGTATACCGCAGGCAATAAAGTGGAT

GTTGATATTCCGACCTTTAATAGCCTGAATACCGATGATTATTAATAAGGCGCGCC

(B)

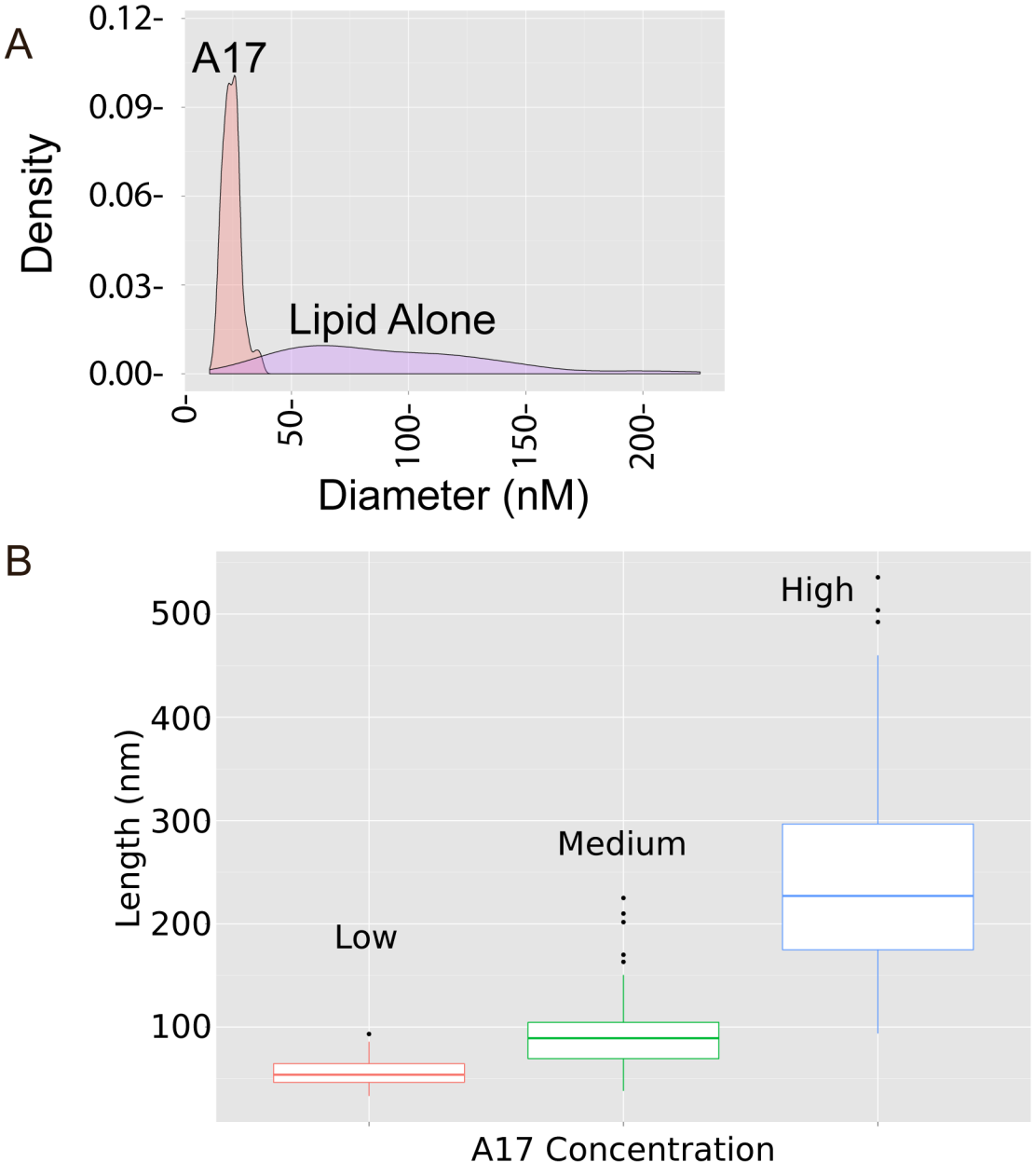
MGSS*HHHHHH* SQDP**LVPRGS** MSYLRYYNML DDFSAGAGVL DKDLFTEEQQ QSFMPKDGGM

MQNDYGGMND YLGIFKNNDV RTLLGLILFV LALYSPPLIS ILMIFISSFL LPLTSLVITY

CLVTQMYRGG NGNTVGMSIV CIVAAVIIMA INVFTNSQIF NIISYIILFI LFFAYVMNIE

RQDYRRSINV TIPEQYTCNK PYTAGNKVDV DIPTFNSLNT DDY

Figure S3


A
A17
Lipid Alone
Density
0.12-
0.09-
0.06-
0.03-
0.00-
0-
50-
100-
150-
200-
Diameter (nM)
B
High
Medium
Low
Length (nm)
500
400
300
200
100
A17 Concentration

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cells, viruses, antibodies

BS-C-1 and RK-13 cells were maintained in Eagle's minimum essential medium or Dulbecco's modified Eagle's medium (Quality Biologicals) supplemented with 10% fetal bovine serum (Quality Biologicals). VACV vTF7-3 and vIndA17 were propagated and titrated as described previously (Fuerst et al., 1986; Wolffe et al., 1996). The A17 antibodies were produced by immunizing rabbits with peptides (Betakova et al., 1999; Wolffe et al., 1996). Anti-protein disulfide isomerase goat polyclonal IgG was from Santa Cruz Biotechnology.

Complementation assay

BS-C-1 cells were infected with 3 plaque forming units (PFU) of vIndA17, an A17 inducible virus in the presence or absence of isopropyl β-d-1-thiogalactopyranoside (IPTG) for 1 h. Cells were then transfected with plasmids using Lipofectamine 2000 (Life Technologies) and the manufacturer's protocol. After infection for 24 h, cells were harvested and frozen and thawed three times before plaque titration on BS-C-1 cells supplemented with IPTG. Plaques were visualized after 24 h by crystal violet staining.

Protein purification

Full length, codon optimized VACV strain Western Reserve A17 (Figure S2, Geneart) with all cysteines mutated to alanines (asterisks in Figure 1A) was cloned into pETDUET-1 (EMD Biosciences) with an N-terminal His_6-tag and a thrombin cleavage site. A17 plasmid was electroporated into C43(DE3) competent cells (Lucigen), recovered for 1 h in SOC medium (Sigma Aldrich), and grown overnight in 150 ml of Luria broth supplemented with 100 µg/ml ampicillin. For expression, 10 ml of cells were transferred to twelve 1 l shaker flasks with 2xYT broth (MP Biomedicals) supplemented with 100 µg/ml ampicillin. Cells were grown to an optical density (600 nm) of 0.8 to 1.0 and induced with 1 mM IPTG and 100 µg/ml Amp for 4 h at 37°C. Cells were pelleted and resuspended in 300 ml TSG (50 mM Tris, 140 mM NaCl, 5% glycerol) before breaking with an EmulsiFlex-C3 (Avestin) twice at 20,000 psi on ice. The membranes were sedimented at 42,000 rpm in a Type 45 Ti Rotor for 1 h at 4˚C. Membranes were solubilized in 1% fos-choline 12 (FC12, Avanti Polar Lipids) for 1 h at 25˚C and detergent soluble A17 was clarified by centrifugation at 42,000 rpm in a Type 45Ti rotor for 45 min at 4˚C. The protein was isolated by Ni-NTA (Qiagen) chromatography and size exclusion chromatography on Superdex 200 10/300 GL (GE Healthcare) in 0.1% FC12. Proteins were concentrated with 30 kDa Amicon Ultra Centrifugal Filters (Millipore). Protein concentration was measured at 280 nm using a Nanodrop (Thermo Scientific).

Proteoliposome formation

From 0.25 to 1.0 mg/ml of A17 (11 to 44 µM final) was reconstituted with 1mg/ml (average 1.25 mM final) *E. coli* polar lipids (Avanti) or 1 mg/ml of a mixture of lipids mimicking the ER (3% DOPS, 18% DOPE, 59% DOPC-average 1.21 mM final) (Avanti). Detergent was removed by dialysis in TS (140 mM NaCl, 5 mM KCl, 25 mM Tris, pH7.4) using a 10-20 kDa cutoff dialysis button (Thermo Scientific) for 3 days at room temperature.

Transfection

Experiments were carried out by expressing A17 in VACV-infected and uninfected cells. For the former, RK-13 or BS-C-1 cells were grown on coverslips in 24-well plates. After reaching 80% confluence the cells were infected with 5 PFU/cell of vTF7-3 and after 1 h were transfected with a plasmid encoding the A17 ORF regulated by a T7 promoter. Lipofectamine 2000 (Invitrogen Life Sciences) was used for transfection according to instructions of the provider. The cells received fresh medium at 6 h after transfection. At 16 h after infection the cells were fixed and prepared for electron microscopy. Alternatively, a BHK-21 cell clone (BSR-T7/5) that expresses T7 RNA polymerase (Habjan et al., 2008) was transfected with the plasmid pVOTE1-A17L, which was derived from pVOTE1 (Ward et al., 1995) and expresses the A17 ORF regulated by the T7 promoter. After 16 h, the cells were fixed and prepared for electron microscopy.

Cross-linking

Methods were modified from Shibata and coworkers (Shibata et al., 2008). Proteoliposomes were used for cross-linking purified A17. For cross-linking A17 in cellular membranes, a 150 cm^2 flask of RK-13 cells was infected with vTF7-3 at a multiplicity of 5 PFU/cell in the presence of AraC (44 µg/ml). After 1 h, the cells were transfected with 12 µg of pVOTE1-A17L. After 21 h, the cells were washed in phosphate

buffered saline and lysed in hypotonic buffer (10 mM HEPES, pH 7.8, 10 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM phenylmethanesulfonyl fluoride) by multiple passages through a 22-gauge needle. The lysate was cleared by low speed centrifugation and then the membrane fraction was obtained by centrifugation at 100,000 x g for 10 min. The pellet was washed once in 25 mM HEPES, pH 7.8, 150 mM potassium acetate, 2.5 mM magnesium acetate, 2 mM phenylmethanesulfonyl fluoride, repelleted and suspended in 60 µl of the above buffer. For both cell-derived membranes and in vitro-derived membranes, samples of 10 µl were treated with 0 to 2 mM EGS at room temperature for 30 min. The reactions were quenched with 2 µl of 1 M Tris pH 7.5, denatured with lithium dodecylsulfate and analyzed by electrophoresis on a 4 to 12% Bis-Tris NuPAGE gel (Invitrogen). The A17 protein was visualized with anti-A17N antibody and donkey anti-rabbit IRDye800CW (Li-Cor).

Maleimide-PEG modification

Cells (1.25×10^5) were infected with 1 PFU/cell of vIndA17 and transfected with the indicated plasmids expressing A17 mutant proteins. After 18 h, cells were harvested by scraping and spinning for 5 min at 2,000 rpm at 4°C. Cells were washed in HCN Buffer (50 mM HEPES, pH 7.3, 150 mM NaCl, 2 mM $CaCl_2$) then resuspended in HCN buffer. Samples were split and combined with digitonin (0.04%) and incubated for 20 min on ice. Solubilized membranes were incubated with maleimide PEG 2000 (Thermo Scientific) for 30 min on ice. The reaction was quenched with addition of 5 mM dithiothreitol and mixed with sample buffer. Samples were analyzed by electrophoresis on a 4 to 12% Bis-Tris NuPage gel (Invitrogen). The A17 protein was visualized with anti-A17N antibody and donkey anti-rabbit IRDye800CW (Li-Cor).

Electron microscopy

For transmission electron microscopy of liposomes, carbon-coated Rh Flashed Copper mesh grids (400 mesh, Ted Pella) with nitrocellulose supporting film were placed on 15 µl droplets of solution for 5 min before negative staining in 1% uranyl acetate. Protein–lipid tube structures were observed and imaged on a FEI Tecnai 12 microscope operating at 120 kV and recorded with a Gatan US4000 CCD camera. NIH Image J software was used for measurements. For immunogold labeling, grids were placed on a 15 µl sample for 5 min, blotted, and then blocked for 30 min in Tris-saline with 1% bovine serum albumin (TSB). The grid was incubated with A17N antibody (1:100 dilution in TSB) for 1 h at 25˚C then washed 3 x 5 min in TSB. The grid was then incubated with protein A-10 nm gold (University Medical Center, Utrecht, Netherlands) at a 1:70 dilution in TSB for 1 h at 25˚C, washed 3 times for 5 min in TSB, then stained with 1% uranyl acetate.

For transmission electron microscopy of thin cell sections, infected cells were fixed with 2% glutaraldehyde and embedded in EmBed-812 resin (Electron Microscopy Sciences, Hatfield, PA). Specimens were viewed with a FEI Tecnai Spirit transmission electron microscope (FEI, Hillsboro, OR).

Tomography

Sections 200-nm-thick were collected on glow-discharged carbon grids, and 10 nm colloidal gold fiducial markers were applied. Using a linear tilt scheme and a Tecnai BioTwin Spirit TEM (FEI) microscope operated at 120 kV, a series of single-axis tilt images were collected. Images captured over a tilt range of ± 60° (1° increments) at a 1-µm defocus level were recorded using an UltraScan 1000 Gatan charge-coupled-device (CCD) camera (2,048 by 2,048 pixels) and automated tomography acquisition software (Xplore 3D; FEI). The resulting images had a binning factor of 1 and a pixel size of 0.9075 nm. Tilt series were then initially aligned using IMOD's BatchRunTomo, and refined in ETOMO before reconstructing weighted back projection tomograms (version 4.8.37). 3-D surface models were created from Gaussian filtered tomograms with a 3-D kernel value of 3 with inverted contrast by thresholding selecting areas of interest using the Amira Visualization Package (version 5.6.0, Visage Imaging, Carlsbad, CA). All manual segmentation was done on unfiltered tomograms with inverted contrast.